DETECTION AND MOLECULAR CHARACTERIZATION OF MYCOPLASMA GALLISEPTICUM AND MYCOPLASMA SYNOVIAE FROM COMMERCIAL CHICKENS IN MALAYSIA

KARTINI AHMAD

FPV 2012 28
DETECTION AND MOLECULAR CHARACTERIZATION OF MYCOPLASMA GALLISEPTICUM AND MYCOPLASMA SYNOVIAE FROM COMMERCIAL CHICKENS IN MALAYSIA

KARTINI AHMAD

MASTER OF VETERINARY SCIENCE
UNIVERSITI PUTRA MALAYSIA

2012
DETECTION AND MOLECULAR CHARACTERIZATION OF *MYCOPLASMA GALLISEPTICUM* AND *MYCOPLASMA SYNOVIAE* FROM COMMERCIAL CHICKENS IN MALAYSIA

By

KARTINI AHMAD

Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Master of Veterinary Science

May 2012
Specially dedicated to:

My dearest parents
who always encourage and support me throughout my study,
Azreen and to all family members.
Abstract of thesis presented to Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Veterinary Science

DETECTION AND MOLECULAR CHARACTERIZATION OF MYCOPLASMA GALLISEPTICUM AND MYCOPLASMA SYNOVIAE FROM COMMERCIAL CHICKENS IN MALAYSIA

By
KARTINI AHMAD
May 2012

Chairman: Professor Datin Paduka Aini Ideris, PhD
Faculty: Veterinary Medicine

Mycoplasma gallisepticum (MG) and Mycoplasma synoviae (MS) are well-known pathogens of poultry, which are distributed world-wide and significantly important for the poultry industry. Both pathogens are capable causing respiratory and joint diseases in chickens and turkeys, subsequently leading to poor production due to poor growth and increase in mortality. Currently, studies on the prevalence of MG and MS, especially on concurrent infections with MG and MS, in commercial chickens as well as genetic diversity of MG and MS isolates are still lacking in Malaysia. Hence, the present study was to detect the presence of MG and MS from commercial chickens (broiler, breeder, layer and village chickens) in Peninsular Malaysia using isolation and molecular methods; and to characterized multigene families of MG (pvpA, gapA, mgc2, pMGA, crmA3 and crmC genes) and MS (vlhA gene) based on gene-targeted sequencing (GTS) analysis. A total of 814 samples of choanal slit and trachea swabs from 27 chicken farms within
Peninsular Malaysia were taken from 472 commercial broiler, 105 broiler breeder, 131 layer and 106 village chickens. *Mycoplasma gallisepticum* was detected in three out of four types of commercial chickens farms (broiler, broiler breeder and village chickens), while MS were detected in all four commercial chickens farms (broiler, broiler breeder, layer and village chickens). Overall, both IFA and PCR methods detected high prevalence of MG infection (9.1% and 24.2%, respectively) than MS infection (2.0% and 5.7%, respectively), while concurrent infection of MG and MS (0.7% and 2.5%, respectively) was the least detected in this study. The prevalence of MG infection and concurrent MG and MS infections were detected higher in broiler chickens followed by broiler breeder, layer and village chickens. *Mycoplasma synoviae* was detected higher in broiler breeder chickens followed by broiler, layer and village chickens. These indices indicate that MG and MS are still persisting in commercial poultry production under current biosecurity and disease control programme. The analysis of multigene families of 20 MG field isolates on six cytadhesin genes revealed their G+C content within 27% - 50% and showed 91-100% sequence homology with MG isolates from USA, Israel, Australia and Russia. Classification of field MG isolates based on a close relationship on nucleotide sequence analysis, sequence similarity identity matrix and phylogeny relations, revealed eleven (11) Malaysian MG field isolates were successfully grouped into 3 categories: 1) S6 strain, 2) ts-11 strain and 3) local field strain. Five isolates (KPR44 L, KPR16W44 L, THNG8W L, PF3H Br and PF7U Br), under local field strain category, were classified as Malaysian isolate as they were more diversified than the reference and published isolates. Analysis on eight MS local isolates
based on size of proline-rich repeat (PRR) region of the \textit{vlhA} gene revealed
gene size polymorphism with 41 - 43\% G+C content and 95 - 98\% sequence
homology with Australian and USA isolates. The lengths of PRR encoding
sequence of local isolates were within 19 - 35 amino acids and the isolates
were classified according to PRR grouping (group A - E). Two field isolates
(ALNH5 BB and LHMN L) were classified in group C with 32 a.a. and one
isolate (JBSJ Br) was classified in group E with 19 a.a. However, five local
isolates (KSVC4 VC, KSVC5 VC, LGW34H9 BB, PPB07 Br and ALNH6 BB)
with 35 amino acid (a.a.) size were not classified within the PRR
classification as there is no classification on 35 a.a. been published or
recorded. These new findings show the unique entity of Malaysian MG and
MS field isolates compared to reference and other published isolates.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains Veterinar

PENGESANAN DAN PENCIRIAN MOLEKULAR MYCOPLASMA GALLISEPTICUM DAN MYCOPLASMA SYNOVIAE DARI AYAM KOMERSIL DI MALAYSIA

Oleh

KARTINI AHMAD

Mei 2012

Pengerusi: Profesor Datin Paduka Aini Ideris, PhD
Fakulti: Perubatan Veterinar

Mycoplasma gallisepticum (MG) dan Mycoplasma synoviae (MS) adalah patogen unggas yang terkenal, yang tersebar di seluruh dunia dan mempunyai kepentingan ketara dalam industri dan ekonomi unggas. Kedua-dua pathogen ini mampu menyebabkan penyakit pernafasan dan sendi pada ayam dan ayam belanda, seterusnya membawa kepada penurunan taraf pengeluaran yang disebabkan oleh penurunan kadar pertumbuhan dan peningkatan kematian. Sehingga kini, kajian prevalens MG dan MS, terutamanya jangkitan serentak MG dan MS, pada ayam komersial serta kepelbagaian perincian genetik isolat-isolat MG dan MS masih kurang di Malaysia. Oleh itu, kajian ini adalah untuk mengesan kehadiran MG dan MS dari ayam komersial (ayam pedaging, ayam pembiak baka pedaging, ayam penelur dan ayam kampung) di Semenanjung Malaysia dengan menggunakan kaedah pengasingan dan molecular; dan mencirikan keluarga multigen MG (gen-gen pvpA, gapA, mgc2, pMGA, crmA dan crmC) dan MS (gen vlhA) berdasarkan analisis jujukan sasaran (GTS). Sebanyak 814
sampel calitan celahan lelangit dan trakea dari 27 ladang ayam di Semenanjung Malaysia telah diambil daripada 472 ayam pedaging, 105 ayam pembiak baka pedaging, 131 ayam penelur dan 106 ayam kampung. *Mycoplasma gallisepticum* telah dikesan pada tiga daripada empat jenis ayam komersial (ayam pedaging, ayam pembiak baka pedaging dan ayam kampung), manakala MS telah dikesan pada kesemua empat jenis ayam komersial (ayam pedaging, ayam pembiak baka pedaging, ayam penelur dan ayam kampung). Secara keseluruhan, kedua-dua kaedah IFA dan PCR mengesakan prevalens jangkitan MG yang tinggi (masing-masing 9.1% dan 24.2%) berbanding jangkitan MS (masing-masing 2.0% dan 5.7%), manakala jangkitan serentak MG dan MS (masing-masing 0.7% dan 2.5%) adalah paling kurang dikesan dalam kajian ini. Prevalens jangkitan MG dan jangkitan serentak MG dan MS dikesan lebih tinggi pada ayam pedaging, diikuti dengan ayam pembiak baka daging, ayam penelur dan ayam kampung. *Mycoplasma synoviae* pula dikesan lebih tinggi pada ayam pembiak baka pedaging diikuti dengan ayam pedaging, ayam penelur dan ayam kampung. Indeks ini menunjukkan MG dan MS masih berterusan dalam produksi ayam komersil di bawah program biosekuriti dan kawalan penyakit yang terkini. Analisis keluarga multigen dari 20 isolat-isolat tempatan MG pada enam gen ‘cytadhesin’ menunjukkan kandungan G+C adalah dalam lingkungan 27-50%, dengan persamaan jujukan homologi antara 91-100% dengan isolat-isolat MG dari Amerika Syarikat, Israel, Australia dan Rusia. Pengkelasifikasi isolat-isolat tempatan MG adalah berdasarkan hubungan rapat pada analisis jujukan nukleotida, keserupaan matriks identiti jujukan dan hubungan filogeni. Ia menunjukkan sebelas (11)
ACKNOWLEDGEMENTS

I would like to extend my gratefulness to almighty Allah for giving me strength and good health that enable me to finish all the research works. My heartfelt gratitude and appreciation to my supervisor, Prof. Datin Paduka Dr. Aini Ideris, who has been very kind and generous to share with me her invaluable knowledge, idea, time, experienced and excellent guidance, support and encouragement throughout my study.

My sincere appreciation and thank you to Prof. Dr. Abdul Rahman Omar, member of the supervisory committee, for his constructive comments, suggestions and proper guidance in my study.

A special thanks to Dr. Tan Ching Giap and other fellow graduate students at the Biologics Laboratory of Faculty of Veterinary Medicine, UPM for their constant encouragement, support, sharing knowledge and friendship along the research study’s journey. You all have been a great help and motivated me to move forward. I would like to thank Siti Khatijah Muhamad, staff at the Biologics Laboratory for her technical assistance and support.

Finally, I would like to express my deepest gratitude to my family for their endless encouragement, sacrifice, patience and understanding. Not to forget to all who have participated in various ways in making my study successful and I am thankful to them all. Thank you.
I certify that a Thesis Examination Committee has met on 18 May 2012 to conduct the final examination of Kartini binti Ahmad on her thesis entitled “Detection and Molecular Characterization of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* from Commercial Chickens in Malaysia” in accordance with the Universities and University College Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The committee recommends that the student be awarded the Master of Veterinary Science.

Members of the Thesis Examination Committee were as follows:

**Saleha binti Abdul Aziz, PhD**
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

**Jalila binti Abu, PhD**
Senior Lecturer
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

**Abdul Rahim bin Abdul Mutalib, PhD**
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

**Zaini Mohd Zain, PhD**
Associate Professor
Faculty of Medicine
Universiti Teknologi Mara
(External Examiner)

\[Signature\]

**ZULKARNAIN ZAINAL, PhD**
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 27 Ogos 2012
This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Veterinary Science. The members of the Supervisory Committee were as follows:

Aini binti Ideris, PhD  
Datin Paduka Professor  
Faculty of Veterinary Medicine  
Universiti Putra Malaysia  
(Chairperson)

Abdul Rahman bin Omar, PhD  
Professor  
Faculty of Veterinary Medicine  
Universiti Putra Malaysia  
(Member)

BUJANG BIN KIM HUAT, PhD  
Professor and Dean  
School of Graduate Studies  
Universiti Putra Malaysia.

Date:
DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or other institutions.

KARTINI AHMAD

Date: 18 May 2012
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>DEDICATION</th>
<th>ii</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>ABSTRAK</td>
<td>vi</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENT</td>
<td>ix</td>
</tr>
<tr>
<td>APPROVAL</td>
<td>x</td>
</tr>
<tr>
<td>DECLARATION</td>
<td>xii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>xiii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xvi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xviii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xx</td>
</tr>
</tbody>
</table>

## CHAPTER

1 GENERAL INTRODUCTION 1

2 LITERATURE REVIEW 7

2.1 Mycoplasmas 7

2.2 History 8

2.3 Avian mycoplasmosis 9

2.4 Incidence and distribution 10

2.5 Transmission 11

2.6 Clinical signs 12

2.7 Pathogenesis 13

2.8 Diagnosis 14

2.8.1 Isolation and identification 15

2.8.2 Serology 16

2.8.3 Molecular methods 17

2.9 The impact of avian mycoplasmosis in poultry production 19

2.10 Intervention strategies 21

2.11 Gene-Target Sequencing analysis of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* multigene families

2.11.1 *Mycoplasma gallisepticum* multigene families 25

2.11.2 *Mycoplasma synoviae* multigene families 28

2.12 Phylogenetic analysis of Mycoplasma 29

3 ISOLATION AND MOLECULAR DETECTION OF *Mycoplasma gallisepticum* AND *Mycoplasma synoviae* FROM COMMERCIAL CHICKEN FARMS 33

3.1 Introduction 33

3.2 Materials and Methods 35

3.2.1 Mycoplasma culture 35

3.2.2 Sampling 35
3.2.3 Isolation and inoculation of Mycoplasma 36
3.2.4 Preparation of MG and MS cultures 37
3.2.5 Detection of Mycoplasma gallisepticum and Mycoplasma synoviae 37
   3.2.5.1 Indirect fluorescence antibody test 37
   3.2.5.2 Polymerase chain reaction 39
       3.2.5.2.1 Total nucleic acid purification 39
       3.2.5.2.2 Quantification of the concentration and purity of the DNA 40
       3.2.5.2.3 Conventional PCR assay 41
   3.2.5.2.4 Detection of PCR products 42
3.2.6 Statistical methods 43
3.3 Results 44
   3.3.1 Detection of Mycoplasma gallisepticum (MG) 44
   3.3.2 Detection of Mycoplasma synoviae (MS) 47
   3.3.3 Concurrent infection of MG and MS in commercial chickens 49
3.4 Discussion 54

4 MOLECULAR CHARACTERIZATION OF MYCOPLASMA GALLISEPTICUM AND MYCOPLASMA SYNOVIAE CYTADHESIN GENES 59
4.1 Introduction 59
4.2 Materials and Methods 62
   4.2.1 Mycoplasma reference strains and isolates 62
   4.2.2 Isolation of genomic DNA 64
   4.2.3 DNA quantification and purity 64
   4.2.4 PCR and oligonucleotides 64
       4.2.4.1 Selection and amplification of MG cytadhesin genes for gene-targeted sequence analysis 64
       4.2.4.2 Amplification of MS vlhA gene 67
   4.2.5 Gel purification of PCR products 68
   4.2.6 DNA sequencing 69
   4.2.7 Sequence and phylogenetic analysis 69
       4.2.7.1 Sequence analysis 69
       4.2.7.2 Phylogenetic analysis 73
4.3 Results 74
   4.3.1 Selection and amplification of MG cytadhesin genes 74
   4.3.2 Amplification of MS vlhA gene 83
   4.3.3 Sequence Analysis of MG cytadhesin gene 84
       4.3.3.1 pvpA gene 84
       4.3.3.2 gapA gene 85
       4.3.3.3 mgc2 gene 85
       4.3.3.4 pMGA gene 86
       4.3.3.5 crmA\(^3\) gene 87
       4.3.3.6 crmC gene 87
   4.3.4 Sequence analysis of MS vlhA gene 91
4.3.5  Phylogenetic tree analysis
   4.3.5.1  MG pvpA phylogenetic analysis
   4.3.5.2  MG gapA phylogenetic analysis
   4.3.5.3  MG mgc2 phylogenetic analysis
   4.3.5.4  MG pMGA phylogenetic analysis
   4.3.5.5  MG crmA phylogenetic analysis
   4.3.5.6  MG crmC phylogenetic analysis
   4.3.5.7  MS vlhA phylogenetic analysis
4.3.6  Classification of MG field isolates

4.4  Discussion
   4.4.1  Mycoplasma gallisepticum
   4.4.2  Mycoplasma synoviae

5  GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATION FOR FUTURE RESEARCH

BIBLIOGRAPHY
APPENDICES
BIODATA OF STUDENT
LIST OF PUBLICATIONS